Available online at www.worldscientificnews.com

World Scientific News

An International Scientific Journal

WSN 145 (2020) 366-378 EISSN 2392-2192

Phytochemical Profile and Antioxidant Potential of Rind Essential Oil of *Citrus reticulate* Blanco

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ABSTRACT

Oxidative stress occurs as a result of an imbalance between production of reactive oxygen species and the regulatory mechanism produced by human cells. The stress contributes to the pathogenesis of inflammatory diseases. Synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and ascorbic acid, are used in curtailing the menace of the stress. Their potential depends on the type of phytochemicals in the oil. It is on this basis this study aimed at isolating, characterizing and investigating antioxidant potential of rind essential oil of *C. reticulata.* To achieve this aim, 500g of fresh rinds of *C. reticulata* were pulverized and hydro-distilled for three hours. GC and GC-MS analysis of the oil revealed the abundance of hydrocarbon monoterpenoids (96%). The most abundant compounds in the oil were D-limonene (82.4%), γ-Terpinene (9.4%) and β-Myrcene (2.2%). The oil exhibited antioxidant activity, with IC_{50}value (10.73µl/ml), which was lower to the activity of ascorbic acid (49.14µl/ml). This shows that the oil is more active than ascorbic acid. Hence, the oil could be explored for the treatment of oxidative stress.

***Keyword*:** Oxidative stress, Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), Phytochemical, Citrus reticulate, Antioxidant

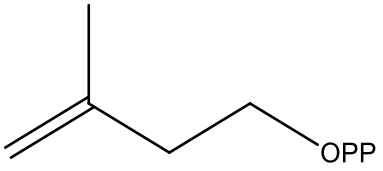
(Received 30 April 2020; Accepted 21 May 2020; Date of Publication 22 May 2020)

1. INTRODUCTION

Essential oils are volatile, natural, complex compounds characterized by a strong odour and are found in aromatic plants (Bakkali *et al*, 2008). The oils are usually colorless, and have a lesser density than that of water. Nevertheless, with age the oil may oxidize which results in the dark coloration. Therefore, essential oil needs to be stored in a cool, dry place tightly stoppered and preferably full in amber glass containers (Hesham *et al*, 2016).

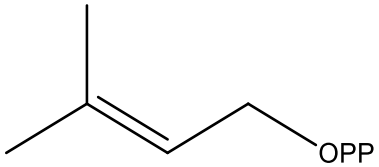
The oils are found in different parts of the plant such as leaves (oregano), seed (almond), flower (jasmine), peel (bergamot), berries (juniper), rhizome (galangal ginger), root (angelica archangelica), bark (sassafras), wood (agar wood), resin (frankincense), petals (rose) (Buchbauer (2000); Burt (2004); Virendra and Diwaker (2007); Hamid *et al*. 2011).

Essential oils play an important role in the protection of plants against herbivores and pathogens. The oils are known for their antiseptic, i.e. bactericidal, virucidal and fungicidal, and medicinal properties and their fragrance; they are used in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetic remedies (Bakkali *et al*, 2008). Essential oils contain several mixtures of volatile secondary metabolites belonging to the terpenoids and phenylpropanoids group (Bakkali *et al*, 2008). The main classes of secondary metabolites that constitute essential oils are monoterpenoids and sesquiterpenoids. The building units for the biosynthesis of these classes of terpenic compounds are isopentenyl diphosphate also known as isopentenyl pyrophosphate (IPP) and dimethylallyldiphosphate also known as dimethylallyl pyrophosphate (DMAPP).



isopentenyl pyrophosphate

(1)



dimethylallyl pyrophosphate

(2)

Figure 1. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP)

Both Isopentenyl pyrophosphate IPP and DMAPP are obtained biosynthetically in plants via mevalonate and non-mevalonate pathways. Essential oils have been isolated from various parts of the plant and different phytochemicals have been identified in the oils. Meanwhile, the most abundant phytochemical in the essential oils of the plant are; α-pinene, D-limonene and γ-Terpinene (Chutia *et al*. 2009; Hicham, 2017).

2. AIM AND OBJECTIVES

The aim of this research is to isolate, characterize and evaluate the antioxidant property of essential oil of rinds of *Citrus reticulata* to develop a more accessible, safer and cheaper antioxidant. The specific objectives to be achieved include:

a) Isolate Essential oil from fresh peels of *Citrus reticulate*
b) Characterize the oil using GC and GC-MS.
c) Determine the Antioxidant potential of the oil.

3. MATERIALS AND METHOD

3. 1. Plant Materials

Ripe fruits of *C. reticulata* were bought from local market in Ilorin, Kwara State, Nigeria. The peels were manually removed. The taxonomic identification of the plant was carried out at the Herbarium of Plant Biology Department, University of Ilorin, Ilorin where the specimen was deposited.

3. 2. Isolation of Essential Oils

Pulverized fresh peels (500g) of *C. reticulate* were hydro-distilled for 3hrs, using Clevenger-type apparatus (with a thermo regulated heating mantle), according to British Pharmacopoeia (1981) specification. The resulting oils were separately collected, preserved in a sealed sample bottle and stored under refrigeration (at 4 ºC) until analysis.

3. 3. Gas Chromatography (GC) Analysis

GC analyses of the oils were performed separately on an Orion micro mat 412 double focusing gas chromatography system fitted with two capillary columns coated with Cp-Sil 5 and Cp-Sil 19 (fused silica, 25 m × 0.25 mm, 0.15 µm film thickness) and flame ionization detector (FID). The volume of each of the oils injected was 0.2 ml, and the split ratio was 1:30. Oven temperature was programmed from 50-230 ºC at 3 ºC/min using hydrogen as a carrier gas. Injection and detector temperature were maintained at 200 ºC and 250 ºC, respectively. Qualitative data were obtained by electronic integration of FID area percent without the use of correction factor.

3. 4. GC and Gas Chromatography-Mass Spectrometry

A Hewlett-Packard HP 5890A GC, interfaced with a VG analytical 70-250s double focusing mass spectrometers was used. The MS operating condition were: ionization voltage 70 ev, ion source and line transfer temperature was maintained at 230 °C. The GC operating conditions were identical, with those of GC analyses.

The MS data were acquired and processed by on-line desktop with a computer equipped with disk memory. The percentage composition of the oil constituents were computed in each case from GC peak areas. The identification of the components was based on the comparison of the retention indices (determined relative to the retention times of series of n-alkanes) and mass spectra with those of authentic samples.

3. 5. Chemicals

All used chemicals were of good analytical grade. The chemicals were supplied by Labtrade (Ilorin, Nigeria). The chemicals used were: DPPH, Methanol and ascorbic acid.

3. 6. DPPH Anti-oxidant Assay

Antioxidant activity of the oil was measured in terms of its hydrogen-donating or radical scavenging ability against DPPH. In the method, 1.5 ml of 2, 2-diphenyl-1-picryl-hydrazil, DPPH, solution (10^{-4} M, in 95% Ethanol) was separately mixed with 1.5 ml of each of the oils at various concentrations (10-50 µl/ml), each of the mixtures was shaken thoroughly and incubated in the dark for 30 minutes at ambient temperature.

The control was prepared using the same procedure without the oil. Absorbance of the solution was measured at 517 nm using UV-spectrophotometer. The assay was carried out in triplicate and the oil that gave 50% inhibition (IC_{50}) was calculated from the graph of the percentage inhibition against the oil concentration. Ascorbic acid was used as a standard.

The percentage inhibitions were calculated using the equation:

$$\text{Inhibition} = \frac{A_0 - A_T}{A_o} \; x \; 100\%$$

where: A_o is the absorbance of the control sample (containing all reagents except the test compound) and A_T is the absorbance of the test samples.

4. RESULTS

Table 1. Percentage Chemical Composition (%) of Essential Oils from Rind of *Citrus reticulate.*

S/N	Compounds	RI	% Composition	MS Data
1	Sabinene	897	0.1	121, 105, **93,** 77, 69
2	β-Pinene	943	0.6	121, 105, **93,** 79, 69
3	β-Myrcene	958	2.2	121, 107, **93,** 79, 69
4	γ-Terpinene	968	9.4	121, 105, **93,** 77,66
5	α-Phellandrene	969	0.1	119, 105, **93,** 77, 65
6	cis-β-Ocimene	976	0.1	121, 105, **93,** 79, 67
7	α-Terpinene	998	0.2	**121**, 105, 93, 77, 65
8	n-Caprylaldehyde	1005	0.3	100, 84, 81, **57**
9	**D-Limonene**	1018	**82.4**	107, 93, 79, **68,** 53
10	o-Cymene	1042	0.5	134, **119,** 103, 91, 77
11	α- Terpinolene	1052	0.4	121, 105, **93,** 79, 67

12	Eucalyptol	1059	0.2	108, 93, 81, 69, **43**
13	β-Linalool	1082	0.8	107, 93, **71**, 69, 55
14	n-Nonaldehyde	1104	0.1	114, 98, 95, 70, **57**
15	Terpinen-4-ol	1137	0.1	111, 93, **71**, 69, 43
16	α-Terpineol	1143	0.1	107, 93, 81, **59,** 43
17	β-Citronellol	1179	0.1	123, 109, 95, 81, **69**
18	Decanal	1204	0.2	110, 95, 70, 57, **43**
19	α –Farnesene	1458	0.6	119, 109, **93**, 79, 69
20	Caryophyllene	1494	0.2	120, 105, **93,** 79, 69
21	α –sinesal	1646	0.4	119, 107, **93,** 79, 66
Total			**99.1**	

Table 2. Percentage Composition of Hydrocarbon Monoterpenoids in the Oil.

S/N	Compounds	R.I	% Composition	Mass Spectra Data
1.	Sabinene	897	0.1	121, 105, **93,** 77, 69
2.	β-Pinene	943	0.6	121, 105, **93,** 79, 69
3.	β-Myrcene	958	2.2	121, 107, **93,** 79, 69
4.	γ-Terpinene	968	9.4	121, 105, **93,** 77,66
5.	α-Phellandrene	969	0.1	119, 105, **93,** 77, 65
6.	cis-β-Ocimene	976	0.1	121, 105, **93,** 79, 67
7.	α-Terpinene	998	0.2	**121**, 105, 93, 77, 65
8.	**D-Limonene**	1018	**82.4**	107, 93, 79, **68,** 53
9.	o-Cymene	1042	0.5	134, **119,** 103, 91, 77
10.	α- Terpinolene	1052	0.4	121, 105, **93,** 79, 67
Total			**95.6**	

Table 3. Percentage Composition of Oxygenated Monoterpenoids in the Oil.

S/N	Compounds	R.I	% Composition	Mass Spectra Data
1.	Eucalyptol	1059	0.2	108, 93, 81, 69, **43**
2.	β-Linalool	1082	0.8	107, 93, **71**, 69, 55
3.	Terpinen-4-ol	1137	0.1	111, 93, **71**, 69, 43
4.	α-Terpineol	1143	0.1	107, 93, 81, **59,** 43
5.	β-Citronellol	1179	0.1	123, 109, 95, 81, **69**
Total			**1.5**	

Table 4. Percentage Composition of Hydrocarbon Sesquiterpenoids in the Oil.

S/N	Compounds	R.I	% Composition	Mass Spectra Data
1.	α –Farnesene	1458	0.6	119, 109, **93**, 79, 69
2.	Caryophyllene	1494	0.2	120, 105, **93,** 79, 69
Total			**0.8**	

Table 5. Percentage Composition of Non-Terpenic Compounds in the Oil.

S/N	Compounds	R.I	% Composition	Mass Spectra Data
1.	n-Caprylaldehyde	1005	0.3	100, 84, 81, **57**
2.	n-Nonaldehyde	1104	0.1	114, 98, 95, 70, **57**
3.	Decanal	1204	0.2	110, 95, 70, 57, **43**
Total			**0.6**	

Table 6. DPPH Radical scavenging Activity of rind essential oil of *Citrus reticulate.*

Percentage Inhibition of DPPH Radical		
Concentration (µl/ml)	Oil	Ascorbic acid
6.25	44.78	41.79
12.5	53.73	46.77
25	56.22	48.26
50	72.14	53.23
100	94.53	66.17

5. DISCUSSION OF RESULTS

5. 1. Yield of Essential Oil from Rinds of *C. reticulata*

The yield of essential oil obtained from rinds of *Citrus reticulate* is 0.52%. The yield was lower to the yield (4.3% w/w). The discrepancy could possibly be attributed to the differences in environmental conditions, which vary from one geographical location to another.

5. 2. Chemical Composition of the Oil

Table 1 shows the chemical composition of essential oil from fresh rinds of *Citrus reticulata*. Twenty one compounds that constituted 99.1% of the oil were identified from their mass spectra.

The percentage composition, retention indices and identities of hydrocarbon monoterpenoids compounds in the oil was shown in Table 2. Ten compounds that represented 96% of the oil were identified from their mass spectra. The most abundant hydrocarbon monoterpenoids was D-limonene (82.4%). γ-Terpinene (9.4%) and β-Myrcene (2.2%) were detected in significant quantities.o-Cymene (0.5%) and β-Pinene (0.6%) were detected in significant amount. Other compounds that were detected in minor quantities were; Sabinene(0.1%), α-Phellandrene (0.1%), cis-β-Ocimene (0.1%), α-Terpinene (0.2%) and α-Terpinolen (0.4%).

Table 3 shows the percentage composition of oxygenated monoterpenoids compounds in the oil. Five compounds that represented 1.5% of the oil were identified. The most abundant oxygenated monoterpenoids from the oil was β-Linalool (0.8%).Eucalyptol (0.2%), Terpinen-4-ol (0.1%), α-Terpineol (0.1%), β-Citronellol (0.1%) and Decanal (0.1%) were detected in minor quantities. The percentage chemical compositions of hydrocarbon sesquiterpenoids in the oil were presented in Table 4. In the table, two hydrocarbon sesquiterpenoids were identified and they represented 0.8% of the oil from their mass spectra. α-Farnesene (0.6%) was detected in significant quantity. Caryophyllene (0.2%) was detected in minor quantity.

The percentage composition of non-terpenic compounds identified in the oil was shown in Table 5. Three compounds that constituted 0.6% of the oil were identified from their mass spectra. All the compounds were detected in minor quantity.

5. 3. Biosynthesis of Monoterpenoids in the Oil

The predominant of D-limonene reveals that the synthase of the most abundant monoterpenoid catalyzed transformation of geranyl pyrophosphate to various cationic intermediates in the presence of divalent metal ions as shown in the reaction Figure 2 below.

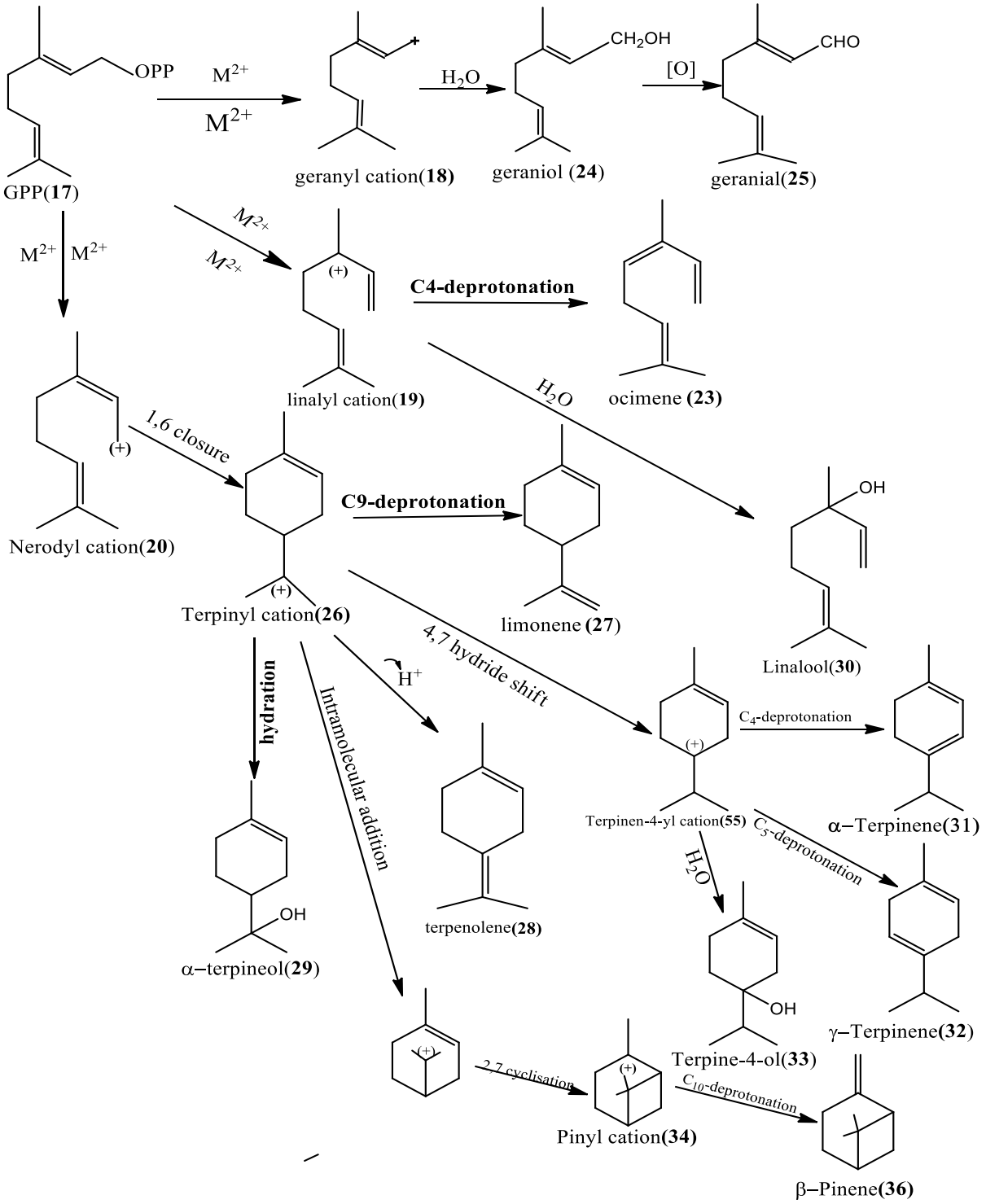


Figure 2. The synthase catalysed ionization of geranyl pyrophosphate to geranyl (**18**), linaly (**19)** and nerody cations (**20**). The C_4-deprotonation of linaly cation (**19**) leads to the formation of ocimene (**23**).Nerody cation (**20**) undergoes6**,** 1-closure of electrophlic addition to give terpinyl cation (**26**). Deprotonation of the ion at C_6 and C_9 produce α-Terpenolene (**28**) and limonene (**27**) respectively. Hydration of the ion forms α-Terpineol (**29**).Terpinyl cation (**26**) undergoes 6, 7 hydride shift to give the terpinen-4-yl cation (**55**). Deprotonation of the ion at C_4 and C_5 will produce α-terpinene (**31**) and γ-terpinene (**32**) respectively. Hydration of the ion produces terpine-4-ol (**31**). Folding of terpinyl cation (**26**) and subsequent electrophilic attack on the C_2=C_3 at the C_2 will form pinyl cation (**34**). Deprotonation of the ion at C_4 and C_{10} will produce α-pinene (**35**) and β-pinene **(36)** respectively.

5. 4. Antioxidant Potential of the Oil

Table 6 shows percentage inhibition of DPPH radical by essential oils from rinds of *C. reticulata.* The activity of the oil was concentration dependent and ranged from 44.78% to 94.53%. At 6.25 µl/ml the activity of the oil was 44.78%.Its activity increased steadily from 53.73% at 12.5 µl/ml to 94.53% at 100 µl/ml. The highest and lowest activity of the oil was obtained at 100µl/ml and 6.25µl/ml respectively.

Similarly, the activity of ascorbic acid against DPPH radical was also in the rangeof 41.79% to 66.17%. The drug has the activity of 41.79% at 6.25 µl/ml. Its activity increased steadily from 46.77% at 12.5 µl/ml to 66.17% at 100 µl/ml. The highest and lowest activity of the drug was obtained at 100 µl/ml and 6.25 µl/ml respectively (Figure 3).

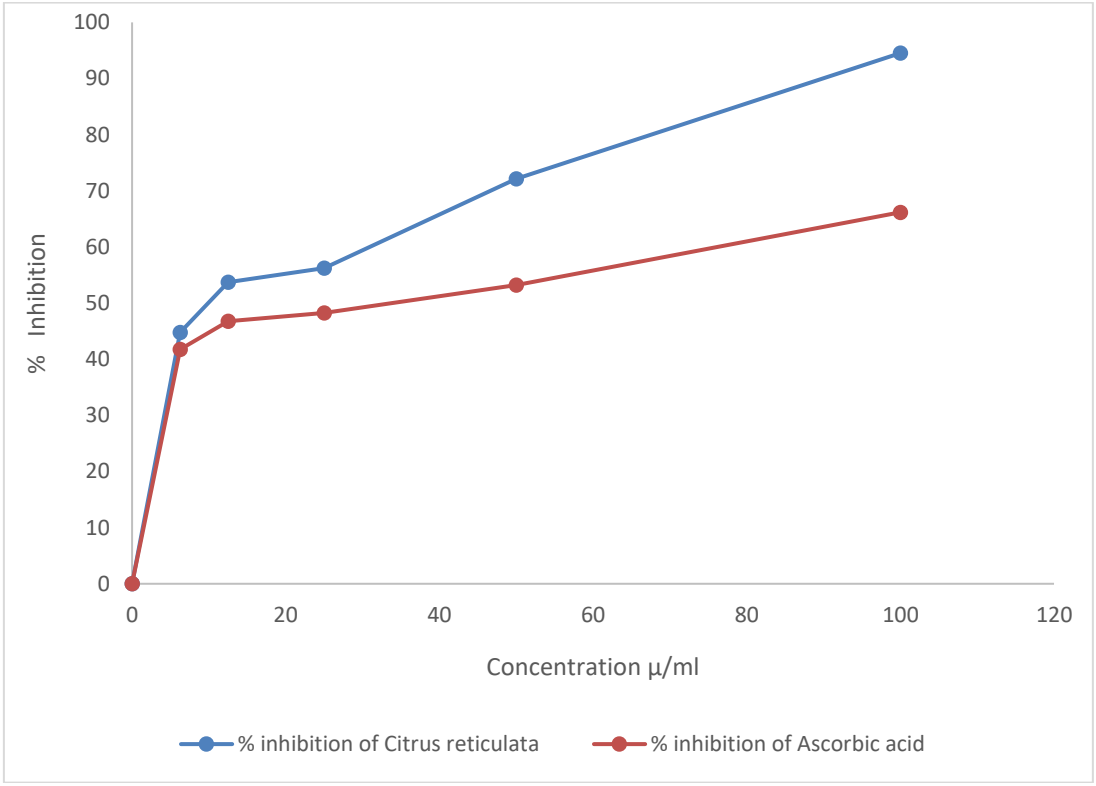


Figure 3. Percentage Inhibition of DPPH Radical by the oil and Ascorbic acid

Table 7 shows the concentration of the oil resulting to 50% inhibition of DPPH radical (IC_{50}). The IC_{50} of the oil, 10.73 µl/ml, was lower than that of the ascorbic acid, 49.14 µl/ml. This revealed that the oil was more active than the ascorbic acid. The higher activity of the oil can be linked to the predominant amount of D-limonene.

Table 7. 50% inhibitory concentration (IC_{50}) of essential oil from rind of *Citrus reticulate* against DPPH and Ascorbic acid as the standard.

Sample	IC_{50}
Essential oil	10.73± 0.39
Ascorbic acid	49.14± 0.034

6. CONCLUSION

The rinds of *Citrus reticulata* yielded 0.52% (w/w) of essential oil and the oil was of D-limonene chemo type. The oil also exhibited antioxidant activity against DPPH radical and its activity was higher than that of ascorbic acid which was used as standard. Hence, the oil could be explored for the treatment of oxidative stress and its complications.

ACKNOWLEDGEMENT

I want to appreciate Almighty God and the efforts of Professor Usman for his supervising of this research and for permission to have access to his laboratory.

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